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(71) Applicant: MEDICAL UNIVERSITY OF SOUTH CAROLINA [US/US]; 171 Ashley Avenue, Charleston, SC 29425 (US).

(72) Inventor: FRANKEL, Arthur, Edward; 1509 Burning Tree Road, Charleston, SC 29412 (US).

(74) Agents: MILLER, Mary, L. et al.; Needle & Rosenberg, 127 Peachtree Street, N.E., Atlanta, GA 30303 (US).

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(57) Abstract

The present invention provides a plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains. In addition, the present invention provides a plant holotoxin fusion protein comprising a moiety consisting of a plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains and a moiety consisting of a ligand specific for a cell surface receptor. Furthermore, a method of constructing a ricin fusion protein immunotoxin is provided, comprising expressing the nucleic acid in a vector in a eukaryotic cell expression system to produce a fusion protein; isolating and purifying the fusion protein; and contacting the fusion protein of with a ricin toxin A chain under conditions which permit the association the fusion protein with the ricin toxin A chain. In addition, the present invention provides a method of treating a cancer or an autoimmune disease in a patient diagnosed with a cancer or an autoimmune disease comprising constructing a ricin fusion protein immunotoxin, wherein the ligand is specific for a particular cell surface receptor present only on the surfaces of the cancer cells or on the surfaces of the cells causing the patient's autoimmune disease; and administering the ricin fusion protein immunotoxin in a pharmaceutically acceptable carrier to the patient, whereby the ricin fusion protein immunotoxin treats the patient's cancer or autoimmune disease.

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METHODS AND COMPOSITIONS FOR RICIN FUSION PROTEIN IMMUNOTOXINS TO TREAT CANCER AND AUTOIMMUNE DISEASE

BACKGROUND OF THE INVENTION

This invention was made with government support under the National Institutes of Health Grant number R01CA54116. The government has certain rights in the invention.

Field of the Invention

The present invention relates to a ricin fusion protein immunotoxin and methods for treating a cancer or autoimmune disease with this immunotoxin. In particular, the present invention provides a ricin fusion protein immunotoxin comprising a ricin toxin B chain from which three lectin binding sites have been removed, conjugated to a ligand specific for a particular cell surface receptor and associated with a ricin toxin A chain. Also provided are methods for administering the ricin fusion protein immunotoxin to treat a cancer or autoimmune disease in a patient diagnosed with a cancer or autoimmune disease.

Background Art

Ricin toxin, a glycoprotein produced in the seeds of *Ricinus communis* plants consists of a galactose-binding B chain (RTB) disulfide linked to an rRNA N-glycosidase A chain (RTA). The 65 kilodalton heterodimeric glycoprotein binds to cell surface galactose-terminated oligosaccharides via lectin binding sites in RTB (1) and undergoes receptor-mediated endocytosis (2). After trafficking to the Golgi (3), the toxin is transported to a distal compartment (4) from which the intersubunit disulfide bond is reduced (5). RTA then translocates to the cytosol and catalytically inactivates protein

synthesis by hydrolysis of a specific adenine base from the 26S ribosomal RNA (6). Galactose binding is important for cell binding and may be needed for internalization and intracellular trafficking of ricin (34).

Ricin toxin is one of the most toxic substances known to man. A single molecule is capable of causing cell death (7), and the LD_{50} of ricin in 20 gram C57/Bl6 mice is reported to be 60 nanograms or 6×10^{11} molecules (8). Histopathological examination of mice given toxic doses of ricin failed to show any definite abnormalities (8). Thus, the critical target organ for ricin is unknown.

The x-ray crystallographic structure of ricin revealed two domains each with three subdomains each with similar folding and primary amino acid sequence (13). While all six subdomains had α-carbon chains forming a loop, twist and hook, only four of the subdomains (1α, 1β, 2α, and 2γ) contained tripeptide kinks. Co-crystallization of 5 mM α-lactose with ricin showed sugar binding in the tripeptide kinks of subdomains 1α and 2γ.

Many patients with hematopoietic malignancies have incomplete responses to chemoradiotherapy and die from progressive disease. Patients' leukemic blasts may develop multiple drug resistance phenotypes and normal tissue toxicities may limit dose escalation.

Fusion toxins have been developed in an effort to treat these diseases. Fusion toxins are hybrid proteins composed of peptide ligands reactive with malignant cells (antibody fragments or cytokines) fused to polypeptide toxins [diphtheria toxin (DT), *Pseudomonas* exotoxin (PE) or ricin]. The toxin-ligand-receptor complex internalizes into intracellular compartments from which the catalytic domain of the toxin translocates to the cytosol and

inactivates protein synthesis. There have been several targets [including interleukin-2 receptors (IL2R), etc.] for which fusion toxins have been designed.

Although ricin fusion toxins have been made, the construction of these toxins has been hampered by the requirement for a reducible disulfide between RTA and the ligand for cell intoxication (30). Initial efforts to produce IL2-RTA fusions yielded nontoxic molecules. Subsequent efforts to introduce a diphtheria toxin loop peptide or factor Xa recognition sequence between IL2 and RTA did not yield disulfide linked molecules and were noncytotoxic to IL2R bearing cells (31). Ricin's extreme potency has led to its use in immunotoxins consisting of monoclonal antibodies chemically coupled to a modified ricin moiety. However, in published clinical trials, the large 200 kilodalton Mr immunoconjugates showed significant vascular endothelial toxicity (32). Three groups of investigators have chemically or genetically modified lectin sites on ricin and used covalently attached ligands to study cell intoxication (33-35). In each case, reductions in lectin function led to profound decreases in cytotoxic potency. However, despite these efforts, novel therapeutic modalities with minimal toxicities and no cross-resistance with current cytotoxic treatments are still needed.

Several other strategies have been used to target the IL2R in patients with leukemias and lymphomas., including antibodies to the α subunit of the interleukin-2 receptor (anti-IL2Rα) conjugated to PE, IL-2 fused to DT and anti-IL2Rα conjugated to RTA. Each of these strategies has yielded only partial success and resulted in some toxic side effects. For example, when RTA was conjugated to anti-IL2Rα and given intravenously to 14 Hodgkin's disease patients, only one partial remission was seen (39). Furthermore, vascular leak syndrome (VLS) with edema, weight gain, hypoalbuminemia and dyspnea was

dose-limiting. In a parallel approach, when IL2 was fused to fragments of DT (18,19) and given systemically to lymphoid malignancy patients, durable complete remissions were rare, suggesting the need for more potent and selective IL2R-directed therapies.

The present invention overcomes previous shortcomings by providing a ricin fusion protein immunotoxin comprising a ricin toxin B chain fusion protein having a modification in a lectin binding site in each of the 1α, 1β and 2γ subunits and a ligand specific for a cell surface receptor in association with a ricin toxin A chain that selectively targets and intoxicates very specific cell populations for the treatment of cancer and autoimmune disease.

SUMMARY OF THE INVENTION

The present invention provides a plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains, For example, a ricin toxin B chain having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains is provided.

In addition, the present invention provides a plant holotoxin fusion protein comprising a moiety consisting of a plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains and a moiety consisting of a ligand specific for a cell surface receptor. For example, a ricin toxin B chain fusion protein comprising a moiety consisting of a ricin toxin B chain having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains and a moiety consisting of a ligand specific for a cell surface receptor is provided.

Furthermore, a method of constructing a ricin fusion protein immunotoxin is provided, comprising expressing the nucleic acid in a vector in a eukaryotic cell expression system to produce a fusion protein; isolating and purifying the fusion protein; and contacting the fusion protein of with a ricin toxin A chain under conditions which permit the association of the fusion protein with the ricin toxin A chain.

In addition, the present invention provides a method of treating a cancer or an autoimmune disease in a patient diagnosed with a cancer or an autoimmune disease comprising constructing a ricin fusion protein immunotoxin, wherein the ligand is specific for a particular cell surface receptor present only on the surfaces of the cancer cells or on the surfaces of the cells causing the patient's autoimmune disease; and administering the ricin fusion protein immunotoxin in a pharmaceutically acceptable carrier to the patient, whereby the ricin fusion protein immunotoxin treats the patient's cancer or autoimmune disease.

Finally provided is a ricin protein immunotoxin comprising a ricin toxin A chain associated with a ricin toxin B chain fusion protein comprising a ricin toxin B chain having a W to S substitution at amino acid position 37 in the 1α subdomain, a Y to H substitution at amino acid position 248 in the 1β subdomain and a Y to H substitution at position 78 in the 2γ subdomain and a ligand specific for a cell surface receptor.

Various other objectives and advantages of the present invention will become apparent from the following description.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention may be understood more readily by reference to the following detailed description of specific embodiments and the Examples included herein. As used in the claims, "a" can include multiples.

The target for several leukemia-directed fusion toxins has been the interleukin-2 receptor (IL2R). IL2R is a heterotrimeric glycoprotein complex on the cell membrane with a 55 kD α subunit, a 75 kD β subunit and a 64 kD γ subunit (16). The only normal human tissues expressing IL2Rα and IL2Rβ are activated T cells, B cells, LGL cells and monocytes and some liver Kupffer cells, lung macrophages and skin Langerhans' cells. Thus, an immunotoxin targeted to this receptor is expected to be reasonably selective. A variety of hematologic neoplasms may show high affinity IL2R expression including hairy cell leukemia, adult T cell leukemia and a fraction of cutaneous T cell lymphomas and B-cell chronic lymphocytic leukemias (17). DT and PE have been fused to either IL2 or antibody Fv anti-IL2R peptides (18-25). All reagents showed potent selective cytotoxicities *in vitro* and in some cases, *in vivo*.

Ricin-based fusion proteins are attractive candidates for development for several reasons. First, the toxin inactivates cell protein synthesis by a mechanism independent of that used by DT or PE. The RNA N-glycosidase activity of ricin cripples 1500 ribosomes/minute and a single molecule of ricin in the cytosol can cause cell death (6,27). Thus, ricin fusion toxins may be used in combination with bacterial fusion toxins or when bacterial fusion toxin resistance is encountered. Furthermore, there is no immunologic cross-reactivity between ricin and the bacterial toxins. Patients who have been immunized with DT or had previous exposure to PE do not show amnestic

immune responses to ricin (28). Finally, there is extensive clinical experience with RTA and blocked ricin immunotoxins suggesting safety in patients (29).

The present invention provides a plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains. The plant holotoxin can be, but is not limited to, ricin, mistletoe toxin, abrin, volkensin as well as any other compound now known or identified in the future to be a plant holotoxin. The preferred plant holotoxin will be characterized as a 65 kD glycoprotein with a galactose lectin B chain and RNA N-glycosidase A chain. The plant holotoxin with the modification described herein can have various functional attributes, such as, for example, the ability to be produced in high yields as an expressed protein in a eukaryotic gene expression system; specific reactivity with antibodies against the plant holotoxin; proper folding to retain the stability and functional characteristics of the wild type holotoxin; proper association with additional moieties which are normally associated with the wild type holotoxin; a 50% lethal dose (LD_{50}) value of greater than ten micrograms in mice; at least a one thousand fold reduction in sugar binding as compared with wild type, at least a one hundred fold reduction in toxicity in mice as compared with wild type; and the ability to selectively intoxicate a target cell with a twenty fold to a greater than two hundred fold reduction in the ability to intoxicate a non-target cell, as compared with wild type. These functional attributes can be determined according to the protocols provided herein in the Examples.

The modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains of the plant holotoxin can be an amino acid substitution, such as, for example, a substitution of an amino acid having an aromatic ring residue with an amino acid lacking an aromatic ring residue. Other modifications in the lectin binding sites can include, but are not limited to, for example, substitution

of an amino acid with a polar hydrogen binding residue with an amino acid with a nonpolar residue, as well as other deletions, additions or amino acid substitutions or any other modifications now known or later discovered that result in either complete or significant removal of the sugar binding activity of the lectin binding site. In the preferred embodiment, the plant holotoxin with modifications will maintain a functional conformation and be able to associate normally with the intoxication-imparting moiety. Whether a given modification results in the complete or significant removal of the sugar binding activity of a lectin binding site can be determined according to the protocols provided in the Examples herein.

Also provided in the present invention is a ricin toxin B chain having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains. The ricin toxin B chain with such modification can have various functional attributes, such as, for example, the ability to be produced in high yields as an expressed protein in a eukaryotic gene expression system; specific reactivity with antibodies against the ricin toxin B chain; proper folding to retain the stability and functional characteristics of the wild type ricin toxin B chain; proper association with ricin toxin A chain; a 50% lethal dose (LD_{50}) value of greater than ten micrograms in mice; at least a one thousand fold reduction in sugar binding as compared with wild type ricin toxin B chain, at least a one hundred fold reduction in toxicity in mice as compared with wild type ricin toxin B chain; and the ability to selectively intoxicate a target cell with a two hundred fold reduction in the ability to intoxicate a non-target cell, as compared with wild type ricin toxin B chain. These functional attributes can be determined according to the protocols provided herein in the Examples.

The modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains in the ricin toxin B chain of this invention can be an amino acid

substitution, such as, for example, wherein the amino acid substitution consists of substitution of an amino acid having an aromatic ring residue with an amino acid lacking an aromatic ring residue. Other modifications in the lectin binding sites can include, but are not limited to, for example, substitution of an amino acid with a polar hydrogen binding residue with an amino acid with a nonpolar residue, as well as other deletions, additions or amino acid substitutions or any other modifications now known or later discovered that result in either complete or significant removal of the sugar binding activity of the lectin binding site. Whether a given modification results in the complete or significant removal of the sugar binding activity of a lectin binding site can be determined according to the protocols provided in the Examples herein.

For example, in the ricin toxin B chain, the amino acid substitutions can consist of a W to S substitution at amino acid position 37 in the 1α subdomain, a Y to H substitution at amino acid position 248 in the 1β subdomain and a Y to H substitution at position 78 in the 2γ subdomain. Other modifications in the lectin binding sites can include, but are not limited to, for example, substitution of an amino acid with a polar hydrogen binding residue with an amino acid with a nonpolar residue, as well as other deletions, additions or amino acid substitutions or any other modifications now known or later discovered that result in either complete or significant removal of the sugar binding activity of the lectin binding site. Whether a given modification results in the complete or significant removal of the sugar binding activity of a lectin binding site can be determined according to the protocols provided in the Examples herein.

A plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in one or more or the subdomains, including at least a modification in the 1β subdomain is also contemplated in the present

invention. The modification in the lectin binding site in one or more of the subdomains can be an amino acid substitution, such as for example, a substitution of an amino acid having an aromatic ring residue with an amino acid lacking an aromatic ring residue (e.g.,a Y to H substitution at amino acid position 248 in the 1β subdomain).

Further provided is a ricin toxin B chain fusion protein having a modification in a lectin binding site in one or more subdomain, including at least a modification in the 1β subdomain. The modification in one or more of the subdomains can be an amino acid substitution, such as, for example, a substitution of an amino acid having an aromatic ring residue with an amino acid lacking an aromatic ring residue. For example, the amino acid substitution can be, but is not limited to, a Y to H substitution at position 248 in the 1β subdomain.

Furthermore, a plant holotoxin fusion protein is provided, comprising a plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains and a ligand specific for a cell surface receptor. The ligand of the plant holotoxin fusion protein can be, but is not limited to, interleukin-2, granulocyte/macrophage colony stimulating factor, an antibody or antibody fragment to CD3, an antibody or antibody fragment to GD2, epidermal growth factor, IGF2, GRF, substance P, MSH, as well as any other molecular entity now known or identified in the future to be a ligand specific for a cell surface receptor as determined by assaying a potential ligand for selective binding avidity for a particular cell surface receptor by protocols standard in the art for measuring binding avidities.

The present invention additionally provides a ricin toxin B chain fusion protein comprising a ricin toxin B chain having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains as described above and a ligand specific for a cell surface receptor. The ligand of the ricin toxin B chain fusion protein can be, but is not limited to, interleukin-2, granulocyte/macrophage colony stimulating factor, an antibody to CD3, an antibody to GD2, epidermal growth factor, an antibody or antibody fragment to CD3, an antibody or antibody fragment to GD2, epidermal growth factor, IGF2, GRF, substance P, MSH, as well as any other molecular entity now known or identified in the future to be a ligand specific for a cell surface receptor as determined by assaying a potential ligand for selective binding avidity for a particular cell surface receptor by protocols standard in the art for measuring binding avidities.

The present invention also provides a ricin toxin B chain fusion protein comprising a ricin toxin B chain having a W to S substitution at amino acid position 37 in the 1α subdomain, a Y to H substitution at amino acid position 248 in the 1β subdomain and a Y to H substitution at position 78 in the 2γ subdomain and a ligand specific for a cell surface receptor. Further provided is a ricin fusion protein immunotoxin comprising this ricin toxin B chain fusion protein associated with a ricin toxin A chain.

A plant holotoxin fusion protein immunotoxin is also provided in the present invention, comprising a plant holotoxin fusion protein (which imparts a binding function to the immunotoxin) as described above, associated with a moiety imparting an intoxicating function to the immunotoxin. For example, a ricin fusion protein immunotoxin is provided in the present invention, comprising a ricin toxin B chain fusion protein consisting of a ricin toxin B chain having a modification in a lectin binding site in each of the 1α, 1β and 2γ

subdomains and a ligand specific for a cell surface receptor, associated with a ricin toxin A chain.

Nucleic acids encoding the plant holotoxins and fusion proteins of this invention are also contemplated, as well as vectors comprising the nucleic acids and hosts comprising the vectors. The present invention also provides nucleic acids complementary to or capable of hybridizing with the nucleic acids encoding the plant holotoxins and fusion proteins.

The nucleic acid of the plant holotoxin or fusion protein can encode the intact plant holotoxin with the modifications described herein or an active fragment thereof. An active fragment of a plant holotoxin is a fragment which is capable of maintaining a functional conformation and associating with the moiety which imparts an intoxicating function to the plant holotoxin. The nucleic acid of the fusion protein can also encode the intact ligand or an active fragment thereof. An active fragment of a ligand of this invention is a fragment which is capable of maintaining a functional conformation and specifically binding to its corresponding cell surface receptor.

Protocols for construction of a vector containing a nucleic acid encoding a plant holotoxin fusion protein such as the ricin toxin B chain fusion protein are well known in the art and are described in the Examples provided herein. The vector can be expressed in any *in vitro* eukaryotic cell expression system, such as, for example, the *Spodoptera frugiperda* insect cell line which expresses proteins in a baculovirus vector, as described in the Examples herein. Isolation and purification of the expressed fusion protein can be carried out by protocols well known to those of skill in the art, e.g., as described in the Examples herein.

Also provided in the present invention is a method of constructing a ricin fusion protein immunotoxin comprising expressing a nucleic acid encoding the ricin toxin B fusion protein, in a vector in a eukaryotic cell expression system, to produce a ricin toxin B chain fusion protein; isolating and purifying the ricin toxin B chain fusion protein; and contacting the ricin toxin B chain fusion protein with a ricin toxin A chain under conditions which permit the association of the ricin toxin B chain fusion protein with the ricin toxin A chain.

The present invention further contemplates a method of constructing a plant holotoxin fusion protein immunotoxin comprising expressing a nucleic acid encoding the plant holotoxin fusion protein described above, in a vector in a eukaryotic cell expression system to produce a plant holotoxin fusion protein; isolating and purifying the plant holotoxin fusion protein; and contacting the plant holotoxin fusion protein with a moiety which imparts an intoxicating function, under conditions which permit the association of the plant holotoxin fusion protein with the intoxicating moiety to yield a plant holotoxin fusion protein immunotoxin.

In addition to the vectors and expression systems described in the Examples herein, a variety of vectors and eukaryotic expression systems such as yeast, filamentous fungi, insect cell lines, bird, fish, transgenic plants and mammalian cells, among others, as are known to those of ordinary skill in the art, can also be used in the present invention.

The vectors of the invention can be in a host (e.g., cell line or transgenic animal) that can express the nucleic acid contemplated by the present invention. As indicated above, the vector, *e.g.*, a plasmid, which is used to transform the host cell, preferably contains DNA sequences to initiate transcription and sequences to control the translation of the protein. These

sequences are referred to as expression control sequences. Suitable vectors for expression systems usually have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, an origin of replication, termination sequences and the like, as desired. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40 virus, adenovirus, bovine papilloma virus, etc, as are well known in the art. For example, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus-type vectors (64). A variety of suitable vectors are described in the literature (see, for example, 65,66).

Appropriate vectors for expressing proteins in insect cells are usually derived from baculovirus. Suitable insect cell lines include, but are not limited to, mosquito larvae, silkworm, armyworm, moth and *Drosophila* cell lines such as a Schneider cell line (67), as well as any other insect cell line now known or identified in the future to be a suitable host cell line for baculovirus or other insect cell expression vectors.

When yeast or higher animal host cells are employed, polyadenylation or transcription terminator sequences from known mammalian genes can be incorporated into the vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (68).

The nucleic acid sequences can be expressed in hosts after the sequences have been operably linked to, i.e., positioned, to ensure the functioning of an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral

part of the host chromosomal DNA. Commonly, expression vectors can contain selection markers, e.g., tetracycline resistance, hygromycin resistance, gentamicin resistance or methotrexate resistance, to permit detection and/or selection of those cells transformed with the desired nucleic acid sequences (*see*, e.g., U.S. Patent 4,704,362). The presence of the vector RNA in transformed cells can be confirmed by Northern blot analysis and production of a cDNA or opposite strand RNA corresponding to the antigen coding sequence can be confirmed by Southern and Northern blot analysis, respectively.

Polynucleotides encoding a variant polypeptide may include sequences that facilitate transcription (expression sequences) and translation of the coding sequences such that the encoded polypeptide product is produced. Construction of such polynucleotides is well known in the art. For example, such polynucleotides can include a promoter, a transcription termination site (polyadenylation site in eukaryotic expression hosts), a ribosome binding site and optionally, an enhancer for use in eukaryotic expression hosts as well as any sequences necessary for replication of a vector.

The host cells are rendered competent for transformation by various means known in the art. There are several well-known methods of introducing DNA into eukaryotic cells. These include, but are not limited to, calcium phosphate precipitation, fusion of the recipient cells with bacterial protoplasts containing the DNA, treatment of the recipient cells with liposomes containing the DNA, DEAE dextran, electroporation, micro-injection of the DNA directly into the cells, as well as any other technique now known or developed in the future for introducing nucleic acid into cells.

The transformed cells are cultured by means well known to one of ordinary skill in the art (69). The expressed polypeptides are isolated from

cells grown as suspensions or monolayers. The latter are recovered by well-known mechanical, chemical, or enzymatic means and purified according to standard methods well known in the art.

Synthesis of heterologous proteins in yeast is well known. For example, Sherman *et al.* (70), is a well-recognized work describing the various methods available to produce a protein in yeast.

Two procedures are used in transforming yeast cells. In one case, yeast cells are first converted into protoplasts using zymolase, lytiacase, or glusulase, followed by addition of DNA and polyethylene glycol (PEG). The PEG-treated protoplasts are then regenerated in a 3% agar medium under selective conditions. Details of this procedure are described by Beggs, J.D. (71) and Hinnen *et al.* (72). The second procedure does not involve removal of the cell wall. Instead, the cells are treated with lithium chloride or acetate and PEG and put on selective plates (73).

Plant holotoxins and fusion proteins, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation and purification techniques to the lysates. The monitoring of the purification process can be accomplished by using Western blot techniques or radioimmunoassay or other standard immunoassay techniques.

The sequences encoding plant holotoxins and fusion proteins of the present invention can also be ligated to various expression vectors for use in transforming cell cultures of, for example, mammalian, insect, plant, bird or fish origin. Illustrative of cell cultures useful for the production of polypeptides are mammalian cells. Mammalian cells permit the expression of proteins in an environment that favors important post-translational modifications such as

folding and cysteine pairing, addition of complex carbohydrate structures and secretion of active protein.

Mammalian cell systems can be in the form of monolayers of cells, although mammalian cell suspensions may also be used. A number of suitable host cell lines capable of expressing intact proteins have been developed in the art, and include the HEK293, BHK21 and CHO cell lines, as well as various human cells such as COS cell lines, HeLa cells, myeloma cell lines, Jurkat cells, etc., as are known in the art. Other animal cells useful for the production of proteins are available, for example, from the American Type Culture Collection Catalogue of Cell Lines and Hybridomas (7th Edition, 1992). Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter (*e.g.*, the CMV promoter, a HSC tk promoter or pgk [phosphoglycerate kinase] promoter), an enhancer (74) and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (*e.g.*, an SV40 large T antigen poly A addition site) and transcriptional terminator sequences.

Efficient post translational glycosylation and expression of recombinant proteins can also be achieved in insect cell expression systems employing baculovirus vectors, as described in the Examples herein.

The plant holotoxin of this invention can also be expressed in transgenic plant expression systems known in the art, such as, for example, soy bean cells or *Nicotiana tabacum* cells (83).

The nucleic acids of the present invention can be used to generate transgenic nonhuman animals in which the nucleic acid encoding a plant holotoxin or fusion protein of the present invention is added to the germ line of

the animal. Thus a cell of the invention containing an nucleic acid of this invention is contemplated to include a cell in a transgenic animal. The plant holotoxin or fusion protein can be isolated and purified from materials secreted by the animal, such as for example, milk secreted from nonhuman mammals. Transgenic animals are generated by standard means known to those skilled in the art (see, for example, 84)

Ricin toxin A chain can be obtained commercially or by expression of a nucleic acid encoding ricin toxin A chain in prokaryotic or eukaryotic *in vitro* expression systems according to standard protocols known in the art and associated with the ricin toxin B chain fusion protein to produce the immunotoxin (82). The nucleic acid encoding the ricin toxin A chain can also be included in a vector which also comprises the nucleic acid encoding the ricin toxin B chain fusion protein of this invention. This vector can be introduced into a eukaryotic expression system, such as a plant cell expression system (83) under conditions whereby the ricin toxin A chain and the ricin toxin B chain fusion protein are produced and associate in the same cell, allowing for isolation and purification of the complete immunotoxin from a single expression system.

As used herein to describe the interactions between ligands and cell surface receptors, interactions between antibodies and antigens or interactions between immunotoxins and target cells, the terms "selective" or "selectively" and "specific" or "specifically" all have the same meaning and are thus used interchangeably to mean either that the ligand binds to only one type of cell surface receptor which has been identified as binding that particular ligand and does not randomly bind to other cell surface molecules, that the antibody binds its corresponding antigen which has been identified and does not randomly bind

with other antigens, or that the immunotoxin binds its intended target cell which has been identified and does not randomly bind to other nontarget cells.

Also contemplated for the present invention is a method for treating or preventing a cancer or an autoimmune disease in a patient comprising constructing the plant holotoxin fusion protein immunotoxin of the present invention as described above, wherein the ligand is specific for a particular cell surface receptor present on the surfaces of the cells to be targeted for intoxication and killing for the purpose of treating or preventing a cancer or autoimmune disease; and administering the plant holotoxin fusion protein immunotoxin in a pharmaceutically acceptable carrier to the patient, whereby the plant holotoxin fusion protein immunotoxin treats or prevents a cancer or autoimmune disease in the patient.

For example, the present invention provides a method of treating or preventing a cancer or an autoimmune disease in a patient comprising constructing the ricin fusion protein immunotoxin of the present invention as described above, wherein the ligand is specific for a particular cell surface receptor present on the surfaces of the cells to be targeted for intoxication and killing; and administering the ricin fusion protein immunotoxin in a pharmaceutically acceptable carrier to the patient, whereby the ricin fusion protein immunotoxin treats or prevents a cancer or autoimmune disease in the patient.

The treatment or prevention of the cancer or autoimmune disease is by the specific intoxication and killing of the cells associated, or potentially associated, with a cancer or autoimmune disease, resulting in prevention of or remission of the cancer, or prevention, elimination or reduction in severity of the symptoms of autoimmune disease. That a given plant holotoxin fusion

protein immunotoxin is effective in treating or preventing a cancer or autoimmune disease in a patient can be determined by evaluating the particular aspects of the medical history, the signs, symptoms and objective laboratory tests that have a documented utility in evaluating disease activity. These signs, symptoms and objective laboratory tests will vary depending on the particular cancer or autoimmune disease being treated or prevented as will be well known to any clinician in this field. Such methods can include, but are not limited to, x-rays, biopsies of biological samples, palpation of masses and measurements of blood and body fluid components. For example, for autoimmune disease (e.g., multiple sclerosis), clinical parameters that can be monitored can include the severity and number of attacks, or for continuously progressive disease, the worsening of symptoms and signs, the cumulative development of disability, the number or extent of brain lesions as determined by magnetic resonance imaging and the need for continued use of immunosuppressive medications (78,79).

The cancer to be treated or prevented by administration of the plant holotoxin fusion protein immunotoxin, such as, for example, ricin fusion protein immunotoxin, can be, but is not limited to, a human leukemia or lymphoma having cancer cells expressing interleukin-2 receptors on the surfaces, wherein the ligand is interleukin-2, acute myelogenous leukemia, wherein the ligand is granulocyte/macrophage-colony stimulating factor and melanoma/neuroblastoma, wherein the ligand is an antibody to GD2, as well as brain neoplasms, epithelial malignancies, sarcomas or any other cancer now known or identified in the future which can be treated or prevented by administration of the plant holotoxin fusion protein immunotoxin of the present invention. Such a cancer would express on the surface of the tumor cells or potential tumor cells an antigen to which the ligand of the present immunotoxin can selectively bind, for selective intoxication of the tumor cells or potential

tumor cells by the ricin protein immunotoxin. In identifying a tumor antigen or potential tumor antigen as a specific target antigen for the immunotoxin of this invention, one of skill in the art would examine various tumor antigens or potential tumor antigens, such as, but not limited to, growth factor receptors, adhesion molecules, oncogene products, differentiation antigens and oncofetal antigens on the surface of a subject's tumor cells or potential tumor cells.

As used herein, autoimmune disease describes a disease state or syndrome whereby a subject's body produces a dysfunctional immune response against the subject's own body components, with adverse effect. The autoimmune disease to be treated or prevented by administration of the ricin protein immunotoxin of the present invention can be, but is not limited to, graft-versus-host disease, wherein the ligand can be an antibody to CD3. Other examples of autoimmune diseases that can be treated or prevented include ulcerative colitis, Crohn's disease, multiple sclerosis, rheumatoid arthritis, diabetes mellitus, pernicious anemia, autoimmune gastritis, psoriasis, Bechet's disease, idiopathic thrombocytopenic purpura, Wegener's granulomatosis, autoimmune thyroiditis, autoimmune oophoritis, bullous pemphigoid, pemphigus, polyendocrinopathies, Still's disease, Lambert-Eaton myasthenia syndrome, myasthenia gravis, Goodposture's syndrome, autoimmune orchitis, autoimmune uveitis, systemic lupus erythematosus, Sjogren's syndrome and ankylosing spondylitis, as well as any other autoimmune disease now known or discovered in the future. Such an autoimmune disease would express an antigen on the surfaces of the cells involved or potentially involved in the autoimmune disease to which a ligand of the present fusion immunotoxin can selectively bind, in order that intoxication of the cells involved or potentially involved in the autoimmune disease can occur.

Also contemplated for the present invention is a method for treating or preventing an allergic disease in a patient comprising constructing the plant holotoxin fusion protein immunotoxin of the present invention as described above, wherein the ligand is specific for a particular cell surface receptor present on the surfaces of the cells involved or potentially involved in the allergic disease, to be targeted for intoxication and killing; and administering the plant holotoxin fusion protein immunotoxin in a pharmaceutically acceptable carrier to the patient, whereby the plant holotoxin fusion protein immunotoxin treats or prevents the patient's allergic disease. For example, the ligand can be the Fc region of immunoglobulin E (IgE).

As used herein, allergic disease describes a disease state or syndrome whereby the body produces a dysfunctional immune response composed of IgE antibodies to environmental antigens and which evoke allergic symptoms. Examples of allergic diseases include, but are not limited to, asthma, ragweed pollen hayfever, allergy to food substances and allergic reactions.

The treatment or prevention of the allergic disease is by the specific intoxication and killing of the cells associated with ,or potentially associated with, the allergic disease, resulting in the prevention of symptoms or the elimination or reduction in severity of the symptoms of the allergic disease. That a given plant holotoxin fusion protein immunotoxin is effective in treating or preventing an allergic disease in a patient can be determined by evaluating the particular aspects of the medical history, the signs, symptoms and objective laboratory test that have a documented utility in evaluating disease activity. These signs, symptoms and objective laboratory tests will vary depending on the particular allergic disease being treated or prevented as will be well known to any clinician in this field. Such methods can include, but are not limited to, x-rays, biopsies of biological samples, palpation of masses and measurements

of blood and other body fluid components. For example, clinical parameters that can be monitored for an allergic disease (e.g., asthma), can include the number and severity of attacks as determined by symptoms of wheezing, shortness of breath and coughing. The measurement of airway resistance by the use of respiratory spirometry, the extent of disability and the dependence on immunosuppressive medications or bronchodilators can also be determined (80,81).

Also contemplated for the present invention is a method of inducing immune tolerance in the patient, comprising constructing the plant holotoxin fusion protein immunotoxin of the present invention as described above, wherein the ligand is specific for a particular cell surface receptor present on the surfaces of certain immune cells to be targeted for intoxication and killing; and administering the plant holotoxin fusion protein immunotoxin in a pharmaceutically acceptable carrier to the patient, whereby the plant holotoxin fusion protein immunotoxin induces immune tolerance. For example, the ligand can be IL2 or an antibody or fragment thereof (e.g., Fv region) to the CD3 antigen.

The induction of immune tolerance is by the specific intoxication and killing of certain immune cells, resulting in the elimination or reduction in severity of a particular immune response. That a given plant holotoxin fusion protein immunotoxin is effective in inducing an immune tolerance in a patient can be determined by evaluating the particular aspects of the medical history, the signs, symptoms and objective laboratory tests that have a documented utility in evaluating immune activity. These signs, symptoms and objective laboratory tests will vary depending on the particular immune response being reduced or eliminated, as will be well known to any clinician in this field. Examples of such methods include, but are not limited to, x-rays, biopsies of

biological samples, palpation of masses and measurements of blood and other body fluid components. In particular, cytokine assays, routine clinical chemistries, immune function assays, complete blood counts and the like, as would be known to the clinician, can be measured at various intervals during treatment.

Additionally, the efficacy of administration of a particular dose of a plant holotoxin fusion protein immunotoxin in preventing a cancer, autoimmune disease, allergic disease, or immune dysfunction requiring the induction of immune tolerance (e.g., transplantation rejection) in a subject not known to have a cancer, autoimmune disease, allergic disease, or immediate need for induction of immune tolerance, but known to be at risk of developing a cancer, autoimmune disease, allergic disease or need for induction of immune tolerance, can be determined by evaluating standard signs, symptoms and objective laboratory tests, as would be known to one of skill in the art, over time. This time interval may be large, with respect to the development of cancer, autoimmune or allergic diseases (years/decades) or short (weeks/months) with respect to the development of a need for induction of immune tolerance. The determination of who would be at risk for the development of a cancer, autoimmune disease, allergic disease or in need of induction of immune tolerance would be made based on current knowledge of the known risk factors for a particular disease or immune response familiar to a clinician in this field, such as a particularly strong family history of disease or need for a transplant.

The plant holotoxins, fusion proteins and immunotoxins of the present invention are preferably provided in a pharmaceutically acceptable carrier and can be parenterally administered to the subject. Suitable carriers for parenteral administration of the immunotoxin in a sterile solution or suspension can

include sterile saline that may contain additives, such as ethyl oleate or isopropyl myristate, and can be injected, for example, intravenously, as well as into subcutaneous or intramuscular tissues.

The plant holotoxin fusion protein immunotoxin can be administered to the subject in amounts sufficient to treat or prevent a cancer, autoimmune disease or allergic disease, or to induce immune tolerance. Optimal dosages used will vary according to the individual, as well as the particular cancer or autoimmune or allergic disease being treated or the type of immune response being induced. Typically, for treatment of humans, plant holotoxin fusion protein immunotoxin, (e.g., ricin toxin fusion protein immunotoxin) would be administered intravenously in a dosage range between 1 μg and 10 mg/kg of body weight and most preferably in a dose of 0.5 mg/kg, either as a single bolus or as a continuous infusion ranging in time from a day to a month. Treatment can be continued for an indefinite period of time, as indicated by monitoring of the signs, symptoms and clinical parameters associated with a particular cancer, autoimmune disease, allergic disease or immune response induction.

The amount of plant holotoxin fusion protein immunotoxin administered will also vary among individuals on the basis of age, size, weight, condition, etc. One skilled in the art will realize that dosages are best optimized by the practicing physician and methods for determining dosage are described, for example, in *Remington's Pharmaceutical Sciences* (77). That a given dosage amount or regimen is effective in treating a cancer, autoimmune disease, or allergic disease or inducing immune tolerance, can be readily determined by using the parameters described above.

The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLES

EXAMPLE I: Construction of a ricin toxin containing a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains.

Selection of RTB modifications. Connolly water-accessible surfaces of RTB subdomains 1β and 2α were plotted using Brookhaven coordinates from Rutenber and Robertus (13) and SYBYL software on a Silicon Graphics Iris Indigo workstation. Aromatic ring residues projecting into the α-carbon kink in each subdomain were identified.

Construction of transfer vectors encoding mutant RTBs. Site-specific mutagenesis was performed on single-stranded pUC119-RTB[W37S/Y248H] DNA using the Sculptor *in vitro* mutagenesis kit as previously described (40). Modifications were made at either the 1β or 2α subdomain to alter aromatic ring residues which provide van der Waals interactions between the protein and sugar. The BamHI-EcoRI mutant RTB encoding DNA fragment was then subcloned into pAcGP67A plasmid (PharMingen, San Diego, CA) and used to transform INVαF' *E. coli* cells. Transfer vectors with mutant RTBs were then purified by cesium chloride-gradient centrifugation.

Isolation of recombinant baculoviruses. pAcGP67A-mutant RTB DNAs (4 μg) were co-transfected with 0.5 μg of BaculoGold AcNPV DNA (PharMingen) into 2 x 10^6 Sf9 *Spodoptera frugiperda* insect cells as recommended by the supplier. On day 7 post-transfection, media were centrifuged and supernatants tested in limiting dilution assays with Sf9 cells as

previously described (40). Positive wells were identified and supernatants reassayed by limiting dilution until all wells up to 10^{-8} dilution were positive. Two rounds of selection were required for each mutant. Recombinant viruses in the supernatants were then amplified by infecting Sf9 cells at a multiplicity of infection (moi) of 0.1, followed by collection of day 7 supernatants.

Expression and purification of mutant RTBs. Recombinant baculoviruses were used to infect Sf9 cells at an moi of 5 in EX-CELL400 media (JRH Scientific, Lexena, KS) with 25 mM α-lactose in spinner flasks. Media supernatants and cell pellets containing mutant RTBs were collected day 6 post-infection. The supernatants were adjusted to 0.01% sodium azide, centrifuged at 3,000x g for 10 minutes, concentrated 15-fold by vacuum dialysis, recentrifuged at 3,000g for 10 minutes, dialyzed into 50 mM NaCl, 25 mM Tris (pH 8), 1 mM EDTA, 0.01% sodium azide and 25 mM α-lactose (NTEAL) at 4°C, ultracentrifuged at 100,000g for one hour at 4°C and loaded onto a P2 monoclonal antibody-acrylamide column. The column was washed with NTEAL and 500 mM NaCl, 25 mM Tris (pH 9), 1 mM EDTA, 0.01% sodium azide, 25 mM α-lactose and 0.1% Tween-20 (NTEALT). Mutant RTBs were eluted with 0.1 M triethylamine HCL (pH 11), immediately neutralized with 1 M sodium phosphate (pH 4.8) and stored at -20°C until assayed. Cell pellets were dissolved in 20 mM Tris HCL(pH 8), 50 mM NaCl, 1% NP40, 1 mM PMSF, 2 ug/ml aprotinin, 1.5 ug/ml pepstatin and 1.5 ug/ml leupeptin, frozen at -70°C, thawed, centrifuged at 22,000g for 15 minutes at 4°C , dialyzed into NTEAL at 4°C and treated identically to dialyzed concentrated cell supernatants.

Chemical and immunological characterization of mutant RTBs. Optical densities at 280 nm were determined. Aliquots were electrophoresed on a reducing 15% sodium dodecyl sulfate (SDS) polyacrylamide gel, stained

with Coomassie Blue R-250 and scanned on an IBAS automatic image analysis system. Aliquots were also electrophoresed on a reducing 15% SDS polyacrylamide gel, transferred to nitrocellulose using a Semi-dry Trans-blot cell (BioRad, Hercules, CA), and immunoblots were performed with rabbit anti-ricin antibody as previously described (40). Antibody enzyme linked immunosorbent assays (ELISA), using monoclonal anti-RTB antibodies P2, P8 or P10 as well coats, were done on each mutant following the method previously reported (15,40).

Lectin activity of mutant RTBs. Asialofetuin ELISAs and experiments to analyze binding to KB cells in the presence or absence of 100 mM α-lactose or 100 ug/ml asialofetuin were performed as previously described (15).

Reassociation of mutant RTBs with plant RTA to form heterodimers. 0.25 ml of mutant RTBs (5 - 15 μg) was mixed with a 4 - fold molar excess of plant RTA (Inland Laboratories) in 0.1 M triethylamine-sodium phosphate pH 7 overnight at room temperature. The reaction mixture was then analyzed by a ricin ELISA utilizing P2 monoclonal anti-RTB coated wells, biotin conjugated αBR12 monoclonal anti-RTA and alkaline phosphatase-conjugated streptavidin detection reagents, as previously described (15).

Cytotoxicity assays. HUT102 cells were incubated with dilutions of ricin and mutant RTB-plant RTA heterodimers at varying concentrations for 24 hours and pulsed for four hours with ^{3}H-leucine as previously detailed (15,40). The 50% inhibitory concentration (IC_{50}) for ricin and each mutant heterodimer was calculated as the concentration which inhibited protein synthesis by 50% as compared with control.

Aromatic ring residues in lectin pocket of subdomains 1β and 2α. The aromatic amino acid residues projecting into the binding cleft were identified to be Tyr-78 and Trp-160. These two residues were selected for mutagenesis to histidine and serine, respectively.

Yields and immunoreactivity of mutant RTBs. Yields were estimated from the optical density at 280 nm of neutralized alkaline eluants post-affinity chromatography (plant RTB OD = 1.44 for 1 mg/ml) and densitometry of Coomassie-stained reducing SDS polyacrylamide gels (10-30% of the protein migrated at 33 kilodaltons). Results were confirmed by densitometry of immunoblots reacted with rabbit anti-ricin antibody. Both triple-site mutants were reactive with the polyclonal antibody. Finally, a monoclonal antibody anti-RTB ELISA was used to verify concentrations of each mutant. All three assays gave similar values. The yield from cell supernatants of the parent double-site mutant, W37S/Y248H, was 205 ug/liter of Sf9 culture. The yield of triple-site mutant, W37S/Y248H/Y78H was 750 ug/liter of culture and the yield of triple-site mutant, W37S/Y248H/W160S was 180 ug/liter of culture. Yields from cell extracts were similar to yields from supernatants for all three mutants.

Reactivities of the mutant RTBs with different monoclonal antibodies to RTB (P2, P8 and P10) were tested by substituting different monoclonal antibodies as capture reagents in the antibody ELISA. Equivalent results were observed for each antibody suggesting similar folding of the mutants. W37S/Y248H RTB reacted 1.3-fold and 4.0-fold more with P8 and P10 monoclonal antibodies, relative to P2 antibody. W37S/Y248H/Y78 RTB reacted 0.7-fold and 1.8-fold as well with P8 and P10 antibodies as with P2 antibody. Finally, W37S/Y248H/W160S RTB bound 1.0-fold and 2.3-fold to P8 and P10, respectively, relative to P2.

Excellent yields of triple-site mutants. The recovery of 0.18 and 0.75 mg triple-site mutant RTB/liter from infected Sf9 cell supernatants was higher than the yields of six single-site RTB mutants and four double-site RTB mutants (15). Further, similar quantities of triple-site mutants were recovered from cell pellets--440 ug/liter for W37S/Y248H/Y78H RTB and 70 ug/liter for W37S/Y248H/W160S RTB. The yields were not dissimilar from the yields for the double-site mutant W37S/Y248H (220 ug/liter from supernatant and 250 ug/liter from cell pellet) or wild-type RTB (400 ug/liter for supernatants) in this expression system and may reflect proper folding for the triple-site mutants. The conservative modification of surface residues (Trp to serine and Tyr to histidine) may have contributed to protein stability.

Immunoreactivity of triple-site mutants. Both triple-site mutants reacted with all monoclonal and polyclonal anti-RTB antibodies tested. Further evidence that these RTB mutants were properly folded included their stability at 4°C and -20°C for one month in 0.1 M triethylamine/sodium phosphate pH 8 and their ability to reassociate with plant RTA.

Sugar binding of mutant RTBs. The double-site RTB mutant, W37S/Y248H, bound asialofetuin 4.8 $\pm$ 2% (n = 6) relative to recombinant or plant RTB. The triple-site RTB mutant, W37S/Y248H/W160S, bound asialofetuin similarly at 1.1 $\pm$ 0.27% (n = 7) relative to plant RTB. In contrast, the triple-site mutant, W37S/Y248H/Y78H, showed minimal to negligible binding to asialofetuin at 0.2 $\pm$ 0.08% (n = 7), relative to plant RTB which was two to three fold higher than background.

An independent measure of mutant RTB binding to glycoproteins was made by detecting mutant RTB bound to cell surfaces. Only W37S/Y248H and W37S/Y248H/W160S showed significant binding to KB cells at 4°C.

Sugar binding of triple-site mutants. W37S/Y248H/W160S RTB retained binding to immobilized asialofetuin and KB cell surface glycoproteins. In both cases, the binding was competed with soluble saccharides. In contrast, W37S/Y248H/Y78H had minimal to negligible sugar binding. These results differ from the findings of Yen and Vitetta with Cos cell-derived mutant RTB (43), Wales and colleagues with Xenopus laevis cell-derived RTBs (44) and Swimmer and colleagues with bacteriophage gene III fusion proteins (45). They reported complete inactivation of sugar binding by modifications of residues in a single subdomain (43) or two subdomains (44,45). However, very small amounts of protein were made and no purification or immunological characterization of the products were done. In each case, decreased sugar binding may have been due, in part, to misfolding or aggregation of recombinant RTBs leading to an overestimation of the effect of their modifications.

Competition experiments. Binding of W37S/Y248H to immobilized asialofetuin was inhibited 3-fold by 100 mM α-lactose and 5-fold by 100 ug/ml asialofetuin. Similarly, W37S/Y248H/W160S binding was inhibited 9-fold by lactose and 27-fold by asialofetuin. Binding of the triple-site mutant W37S/Y248H/Y78H was minimally inhibited by either lactose or asialofetuin--3-fold in each case. Binding of the double-site mutant and 1α, 2α, 2γ triple-site mutant to KB cells was blocked by 100 ug/ml asialofetuin.

Heterodimer formation. Incubation of 5×10^{-7} M - 2.5×10^{-6} M mutant RTBs with excess plant RTA overnight at room temperature led to 70% reassociation of W37S/Y248H, 24% reassociation of W37S/Y248H/Y78H and 57% reassociation of W37S/Y248H/W160S. Similar levels of reassociation were seen using plant RTB or recombinant wild-type RTB with plant RTA under the same conditions. The heterodimer concentrations were quantitated

by an ELISA which identified molecules with both RTB and RTA epitopes and by densitometry of 65 kilodalton bands of immunoblots with anti-RTB and anti-RTA antibodies. Both ELISA and immunoblots gave similar values and showed both mutants reassociated well with plant RTA and had minimal homodimer formation.

Cytotoxicity of mutant heterodimers. The IC_{50} of ricin on HUT102 human leukemia cells was 4×10^{-12} M. The IC_{50} for W37S/Y248H was 2×10^{-10} M; the IC_{50} for W37S/Y248H/W160S was 1×10^{-10} M; and the IC_{50} for W37S/Y248H/Y78H was 5×10^{-9} M. Plant RTA alone had a 20-fold higher IC_{50} of 10^{-7} M.

Cell intoxication functions of the triple-site mutants. Cell sensitivity to mutant heterodimers paralleled mutant residual sugar-binding activity. Significant residual potency was seen with the 1α,2γ and 1α,2α,2γ mutant heterodimers. Their IC_{50}'s were at least two and one-half logs lower than plant RTA alone (RTB alone was nontoxic with $IC_{50} > 6 \times 10^{-6}$M). In contrast, the 1α,1β,2γ mutant RTB-RTA had minimal toxicity above background.

Widely separated ricin lectin sites. The 1α and 2γ sites are separated by 36 Angstroms. 1β and 2γ sites are 44 Angstroms apart. The 1α and 1β distance is 19 Angstroms. These inter-binding site distances are much larger than the inter-site spacing for the hepatic Gal/GalNac receptor and its triantennary N-glycoside ligand (10 -20 Angstroms) (41). Instead, the ricin geometry resembles the spacing of sites on surface binding lectins including mammalian mannose-binding protein, influenza virus hemagglutinin, pertussis toxin and cholera toxin (42). These proteins are phylogenetically unrelated based on lack of primary or tertiary structure homology. Nevertheless, in all these proteins, the sugar combining sites are multiple widely spaced and project

toward a single plane. Thus, they are ideally suited for binding to eukaryotic cell surfaces.

Three RTB lectin sites. The three binding sites on ricin may provide the optimal geometry for binding to the uneven galactosyl oligosaccharide-rich surface of mammalian cells similar to camera tripods or stools. The RTB 1γ and 2β subdomains are unlikely to contribute additional sugar binding as they lack the tripeptide α-carbon kink, aromatic residues or charged residues for hydrogen bond formation. Further, no RGD-like domains exist in RTB--unlike discoidin I from the slime mold, *Dictylostelium discoideum* (46).

EXAMPLE II: *In vivo* toxicity of a ricin toxin containing a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains.

Ricin and recombinant heterodimers. Purified castor bean ricin (3.9 mg/ml in 0.15 M NaCl, 0.015 M potassium phosphate pH 7, 0.1% sodium azide) was purchased from Sigma (St. Louis, MO). Purified deglycosylated RTA (5 mg/ml in PBS) was a gift of Dr. Jerry Fulton, Inland Laboratories, Dallas, Texas. Partially purified recombinant mutant RTBs were prepared and reassociated with plant RTA as previously described (15,40). The proteins tested and their characteristics are shown in Table 1. Ricin was stored at 4°C and all other tested proteins were stored at -20°C until used.

Mice. Pathogen-free C57B/6 female mice (16-18 grams) were purchased from Jackson Laboratories (Bar Harbor, ME) or Harlan Sprague-Dawley (Indianapolis, IN), culled on each experiment to obtain 24 animals each 18 ± 1 grams, and housed in groups of four in specific pathogen-free environment in Micro-Isolator cages (Lab Products, Maywood, NY).

Experimental Design. Mice were injected intraperitoneally with dilutions of toxins in phosphate buffered saline (PBS) plus 0.5% bovine serum albumin (BSA) so that each animal received 0.25 - 0.5 milliliters solution. Animals were then observed twice daily for mortality. Six different concentrations were tested on groups of four mice each for each protein. Graphs of animal survival versus time were prepared for each protein. Once the LD_{50} was determined for each protein, single animals received the LD_{50} and were collected at death or when severely morbid and autopsies were performed.

Chi square contingency tables were formulated for groups of doses for each toxin as described (47).

Histology protocols. Postmortem examinations were carried out on mice given LD_{50} doses on moribund animals. Samples from lungs, thymus, heart, esophagus, trachea, lymph node, liver, spleen, pancreas, kidneys, adrenals, gallbladder, stomach, duodenum, jejunum, colon, ovaries, uterus, brain, spinal cord, and skeletal muscle were taken for microscopic examination. The tissues were fixed in 4% buffered formaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Ricin wild-type, mutants, and subunits. The ricin proteins tested for *in vivo* toxicity are listed in Table 1. Examples of wild-type ricin and ricins with modifications of one, two or three sites were used. The insect-derived RTB mutants had similar folding to plant RTB based on yield from insect cells, migration on SDS-PAGE, immunoreactivities with polyclonal and monoclonal antibodies, CD spectra, protease sensitivity and ability to reassociate efficiently with plant RTA, as described above. The concentration of recombinant heterodimers was based on a sandwich ELISA previously described (15).

Mouse lethality of ricin proteins. Castor bean ricin led to death of all four mice receiving 500 ng three days post-injection. Three of four mice receiving 100 ng died on days 5, 7 and 8 post-injection. None of the mice receiving 1, 5, 10 or 50 ng ricin died. Thus, higher doses led to earlier animal deaths. The LD_{50} was estimated as 75 ng.

Mice treated with 130 ng, 650 ng, 1.3 μg, or 2.6 μg all died after injection with RTB[Y248H]-RTA (2γ single-site mutant heterodimer). Three of four mice treated with 2.6 μg died on day 2 and one of four mice died on day 4 post-injection. Three of four mice treated with 1.3 μg died on day 3 and one of four died on day 5. One of four mice receiving 650 ng died on day 4 and the rest died on day 5. The 130 ng treated mice died on day 5 (1 mouse), day 6 (2 mice) and day 7 (1 mouse). Three of four mice treated with 260 ng died on day 6 (one mouse) and day 7 (two mice). None of the mice treated with 65 ng died. Similar to wild-type ricin, higher doses led to animal deaths on day 3 and 4 post-injection, while the doses closer to the LD_{50} caused death on days 5 - 7. The estimated LD_{50} was 100 ng.

Mice given RTB[W37S/Y248H]-RTA (1α/2γ double-site mutant heterodimer) tolerated more toxin than animals receiving wild-type or single-site mutant ricin. One of four animals died on day 1 and 3/4 animals died on day 2 post-injection with 10 μg. Three of four animals died on day 1 post-injection of 5 μg. Three of four animals died on day 2 and one of four died on day 3 post-2 μg injections. The 1 μg dose killed two mice--one each on day 4 and 5. Similarly, the 500 ng dose led to the death of two mice--one each on days 7 and 10. None of the mice receiving 100 ng died. Again, animals treated with doses much higher than the LD_{50} died on days 1-3, while animals treated with lower toxic doses died on days 4 - 10. The LD_{50} was estimated at 500 ng.

Only limited toxicity was observed with RTB[W37S/Y248H/Y78H]-RTA (1α/1β/2γ triple-site mutant heterodimer). None of the mice receiving 0.1 μg, 0.5 μg, 1 μg, 2 μg, or 5 μg died and only one of four mice receiving 10 μg died on day 3 post-injection. Thus, the LD_{50} was estimated at >10 micrograms.

Plant RTA was significantly less toxic than either the wild-type ricin or mutant heterodimers.

Using chi square contingency tables, the survival rates of mice treated with RTA and the triple-site mutant ricin were calculated to be significantly different ($p<0.001$) than the survival rates of wild-type, single-site mutant or double-site mutant ricins over a range of doses.

The relationship of time to death post-toxin infusion with the ratio dose/LD_{50} for the ricin proteins was also examined. A linear regression analysis showed the correlation was of borderline significance with an $r^2 = 0.48$. Previous investigators have demonstrated earlier deaths at higher toxin doses (52).

The relationship of toxic lectin Kd or dissociation constant for asialofetuin binding with the LD_{50} was also analyzed. A striking correlation was seen with an r^2 of 0.959. Thus, over 95% of the variation in LD_{50} could be explained by sugar binding avidity.

Histology results. The mice treated with intraperitoneal ricin or RTA showed apoptosis in the thymus and spleen. Peritoneal inflammation was observed in some animals presumably due to intraperitoneal injection of toxin. There was no evidence of apoptosis based on nuclear morphology in the other

organs examined. The triple-site mutant heterodimer treated mouse showed no histopathologic abnormalities.

The critical target tissue of ricin in animals is unknown. Previous histopathologic examination similarly failed to reveal differences from untreated animals at either the light or electron microscopic level (8). Protection of mice by intracerebral injection of anti-ricin antibodies led to the hypothesis that the brain is the lethal target for ricin (48), however very little radiolabeled ricin was shown to cross the blood-brain barrier and reach the central nervous system (49). No evidence of central nervous system damage was found in this histological study. Nevertheless, the observation in the present study that modification of lectin sites reduces *in vivo* toxicity supports the role for galactoside receptors on cells in the critical target organ.

The finding of lymphoid apoptosis after ricin administration might suggest that those cells have a lower apoptotic threshold. Deletion of lymphocytes is an important ongoing process for tolerance induction and immune regulation in the lymphoid system (50).

The toxicity of RTA alone may be due to the presence of small amounts (<0.1%) of contaminating ricin. Histopathology in this study did not show extensive renal injury previously reported by others with membranous glomerulopathy, periglomerular fibrosis and tubular hyaline casts (51). In the study by Soler-Rodriguez, hypoalbuminemia and weight gain were also demonstrated. These findings were compatible with a vascular endothelial injury. However, reproducible histologic evidence of endothelial or vascular damage was not demonstrated in this study.

The effect of modification of a third RTB subdomain on ricin toxicity was dramatic with a > 20-fold increase in LD_{50}.

EXAMPLE III: Construction of a ricin fusion protein immunotoxin containing a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains.

Construction of plasmid. Site-specific mutagenesis was performed on single-stranded pUC119-RTB[W37S/Y248H] DNA using the Sculptor *in vitro* mutagenesis kit (Amersham, Arlington Heights, IL) as previously described (40). The aromatic ring residue Tyr-78 in the 1β subdomain was changed to histidine to reduce van der Waals interactions between the protein and galactosides. The BamHI-EcoRI mutant RTB encoding DNA fragment was subcloned into pAcGP67A plasmid (PharMingen, San Diego, CA) and used to transform INVαF' *E. coli* cells (InVitrogen, San Diego, CA). Transfer vector with mutant RTB was then purified by cesium chloride density centrifugation, restricted with BamHI, bound and eluted from silica matrix (Promega, Madison, WI), digested with calf intestinal phosphatase (Boehringer-Mannheim, Indianapolis, IN), heat inactivated and repurified on silica matrix. The BamHI fragment encoding IL2 prepared by polymerase chain reaction of pDW27 plasmid DNA as previously described (54) was isolated from pUC119-IL2 by digestion of cesium chloride density gradient purified plasmid with BamHI, agarose electrophoresis and binding and elution from silica matrix. The 406 bp fragment was subcloned into pAcGP67A-ADP-RTB[W37S/Y248H/Y78H]. The expression vector was maintained in INVαF' *E. coli* using 100 ug/ml ampicillin. Plasmid isolated by alkaline lysis followed by cesium chloride density gradient centrifugation was double-stranded dideoxy sequenced by the Sanger method (33) using the Sequenase kit (USB, Cleveland, OH).

Expression of fusion toxin. Sf9 *Spodoptera frugiperda* ovarian cells (2 x 10^6), maintained in TMNFH medium supplemented with 10% fetal calf serum and 50 ug/ml gentamicin sulfate, were co-transfected with pAcGP67A-ADP-IL2-ADP-RTB[W37S/Y248H/Y78H] DNA (4 μg) and 0.5 μg of BaculoGold AcNPV DNA (PharMingen) following the recommendations of the supplier. At 7 days post-transfection, medium was centrifuged and the supernatant tested in a limiting dilution assay with Sf9 cells and dot blots with random primer ^{32}P-dCTP labeled RTB DNA as previously described (53). Positive wells were identified and supernatants reassayed by limiting dilution until all wells up to 10^{-8} dilution were positive. Two rounds of selection were required. Recombinant virus in the supernatant was then amplified by infection Sf9 cells at an moi of 0.1, followed by collection of day 7 supernatants. Recombinant baculovirus was then used to infect 2 x 10^8 Sf9 cells at an moi of 5-10 in 150 ml EXCELL400 medium (JRH Scientific, Lexena, KS) with 25 mM lactose in spinner flasks. Media supernatants containing ADP-IL2-ADP-RTB[W37S/Y248H/Y78H] were collected at day 6 post-infection. Three different preparations were made.

Protein purification. Media supernatants were adjusted to 0.01% sodium azide and maintained through all purification steps at 4°C. The supernatants were concentrated 15-fold by vacuum dialysis, centrifuged at 3,000x g for 10 minutes to remove precipitate, dialyzed against 50 mM NaCl, 25 mM Tris (pH 8), 1 mM EDTA, 0.01% sodium azide and 25 mM lactose (NTEAL), ultracentrifuged at 100,000g for one hour and bound and eluted from a P2 monoclonal antibody-acrylamide matrix as previously described (53). P2 is an anti-RTB monoclonal antibody. The affinity matrix was prepared using Ultralink azlactone functionality bis-acrylamide following the recommendations of the manufacturer (Pierce, Rockford, IL). Recombinant protein was absorbed to the column in NTEAL, washed with 0.5 M NaCl, 25

mM Tris pH 9, 1 mM EDTA, 0.1% Tween 20, 0.02% sodium azide, 25 mM lactose and eluted with 0.1 M triethylamine hydrochloride (pH 11). The eluant was neutralized with 1/10 volume 1 M sodium phosphate pH 4.25 and stored at -20°C until assayed. Three preparations were made.

Characterization of recombinant protein. Total protein concentration of the affinity column eluant was measured by absorbance at 280 nm. Since the optical densities of a 1 mg/ml solution of RTB and IL2 were 1.4 and 0.7, respectively, a 1 mg/ml solution of fusion protein should have a mass average optical density of 1.16. Protein was also quantitated by BioRad protein assay as per recommendations of the supplier. Aliquots of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H], plant RTB and prestained low molecular weight standards were electrophoresed on a reducing 15% SDS polyacrylamide gel, stained with Coomassie Blue R-250 and scanned on an IBAS automatic image analysis system (Kontron, Germany). Immunological analysis was performed using both an ELISA and immunoblot format. Costar EIA microtiter wells were coated with 100 µl of 5 ug/ml of monoclonal antibody P2, P8, or P10 reactive with RTB or monoclonal antibody to IL2, washed with PBS plus 0.1% Tween 20, blocked with 3% BSA, rewashed and incubated with samples of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H], human IL2 or plant RTB, rewashed, reacted with 1:400 rabbit antibody to ricin or 1:500 rabbit antibody to IL2, washed again, incubated with 1:5000 alkaline phosphatase conjugated goat anti-(rabbit IgG), rewashed, developed with 1 mg/ml p-nitrophenylphosphate in diethanolamine buffer (pH 9.6) and read on a BioRad 450 Microplate reader at 405 nm. Aliquots of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H], bacterial IL2, recombinant RTB, plant RTB, and prestained low molecular weight protein standards were run on a reducing 15% SDS-PAGE, transferred to nitrocellulose, blocked with 10% Carnation's nonfat dry milk/0.1% bovine serum albumin (BSA)/0.1% Tween 20, washed with PBS

plus 0.05% Tween 20, reacted with either 1:400 rabbit antibody to ricin or 1:100 mouse monoclonal antibody to IL2 (5 ug/ml), rewashed, incubated with alkaline phosphatase conjugated goat anti-(rabbit IgG) or anti-(mouse IgG), washed again and developed with the Vectastain alkaline phosphatase kit.

RTA Reassociation protocols. 30 μg of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H] was mixed with 100 μg of plant RTA in a total volume of 0.5 ml of 0.1 M triethylamine/0.1 M sodium phosphate (pH 7) and shaken overnight at room temperature. The reaction mixture was then analyzed by a modified ricin ELISA as previously described (15). Reassociated mixtures were also analyzed by non-reducing SDS/PAGE followed by immunoblots with P2 and P10 anti-RTB monoclonal antibodies (10 ug/ml each), monoclonal antibody to IL2 (5 ug/ml) or monoclonal antibody αBR12 to RTA (10 ug/ml) . Densitometric scanning with the automatic image analysis system was done to quantify the shift of immunoreactive material from 50 kDa to 80 kDa.

Lectin activity assay. Asialofetuin (1 ug/ml) was bound to Costar EIA plate wells and an ELISA was performed as previously detailed with samples of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]-RTA and castor bean ricin (15) Briefly, the asialofetuin coated wells were washed with PBS plus 0.1% Tween 20, blocked with 3% BSA, rewashed and incubated with 12 different concentrations of samples in EX-CELL400, rewashed and reacted with 100 μl of biotinylated αBR12 monoclonal anti-RTA antibody, rewashed and incubated with streptavidin-alkaline phosphatase, washed again and developed with p-nitrophenylphosphate in 50 mM diethanolamine (pH 9.6). Absorbance of wells was measured at 405 nm on a microtiter plate reader. The concentration of protein giving half-maximal binding (Kd) was calculated.

IL2 receptor binding specificity assay. HUT102 human T leukemia cells bearing the high affinity IL2 receptor, YT2C2 human leukemia cells bearing the intermediate affinity IL2 receptor, MT-1 human leukemia cells bearing the low affinity IL2 receptor and CEM human leukemia cells and KB human epidermoid carcinoma cells lacking the IL2 receptor were washed with PBS and attached to polylysine-coated tissue culture dishes and centrifuged at 2000g for 10 minutes. The IL2R content of these cells was previously determined (15). The cells were then incubated live at 4°C. The cells were washed with 2 mg/ml BSA in PBS and incubated in PBS plus BSA with 1 ug/ml castor bean ricin or IL2-lectin-deficient-ricin. The incubation was done at 4°C. The cells were then washed with PBS and incubated with αBR12 mouse monoclonal antibody to RTA (5 ug/ml) plus BSA for 30 minutes at 4°C. The cells were then washed with PBS and reacted with goat anti-(mouse Ig) conjugated to rhodamine (Jackson ImmunoResearch, West Grove, PA) at 25 ug/ml for 30 minutes at 4°C. The cells were washed again in PBS and fixed in 3.7% formaldehyde in PBS, mounted under a #1 coverslip in glycerol-PBS (90:10) and examined using a Zeiss Axioplan epifluorescence microscope.

Cytotoxicity assay. Measurement of protein synthesis inhibition by ricin and ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]-RTA in cultured cells was done as previously described using HUT102, CEM, YT2C2, MT-1, and KB cells (15). ADP-IL2-ADP-wild-type RTB-RTA and ADP-IL2-ADP-RTB[W37S/Y248H] prepared as described previously were also tested.[18,22] All assays were performed in triplicate. Twelve different concentrations of toxins were used. The ID_{50} was the concentration of protein which inhibited protein synthesis by 50% compared with control wells without toxin. There was no purification step after heterodimer reassociation. The free RTA concentration at the highest concentration of heterodimer in the assay (5×10^{-7}M) was 10^{-7}M. On all five cell lines, the ID_{50} for free RTA was $2 - 3 \times 10^{-6}$ M.[28] Thus, in the

range of heterodimer ID_{50}'s (10^{-8} M - 10^{-12} M), the free RTA concentration (2 x 10^{-9} M - 2 x 10^{-13} M) should not produce cytotoxicity.

Blocking of cytotoxicity with IL2 or lactose. HUT102 cells (1.5 x 10^4) were placed in sterile Eppendorf tubes at 4°C in 100 µl leucine-poor RPMI 1640 + 10% dialyzed fetal calf serum with or without 20 ug/ml IL2 or 60 mM α-lactose. Dilutions of IL2-lectin-site modified ricin and ricin at varying concentrations were added in identical medium with or without IL2 or lactose and incubated at 4°C for 30 minutes. Cells were pelleted at 2000g for five minutes, washed once with leucine-poor RPMI 1640 + 10% dialyzed fetal calf serum, resuspended in 150 µl of the same medium and incubated at 37°C in 5% CO_2 for 24 hours. ^{3}H-leucine was added as above and cells were harvested four hours later with a Skatron cell harvester and ^{3}H-leucine incorporation was measured in a liquid scintillation counter. Blocking of selective cytotoxicity was estimated by comparing the ID_{50} of toxins in the presence or absence of IL2 or lactose.

Yield and purity of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]. Three individual preparations from 100 ml cell supernatants were partially purified. Peak eluant fractions contained 186 µg, 228 µg and 167 µg protein based on absorbance at 280 nm. BioRad protein assay gave values of 120 µg, 160 µg, and 140 µg, respectively for the three preparations, using a BSA standard. Densitometry of Coomassie stained gels showed only a single detectable band at 50 kDa in each preparation. However, P2 antibody ELISA showed the concentration of anti-RTB immunoreactive protein was 102 µg, 49 µg, and 75 µg, respectively. Thus, purity was between 21 and 85 % based on absorbance, BioRad protein assay and densitometry of Coomassie-stained gels.

Immunologic cross-reactivity of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]. Different monoclonal antibodies to RTB (P2, P8 and P10) and an antibody to IL2 reacted similarly with the fusion molecule based on antibody capture ELISA. The concentration of the fusion molecule was based on a comparison of P2 antibody binding with plant RTB, so that its relative binding is taken as 100%. Antibody P8 bound 100 % as well with the fusion molecule as with plant RTB. Antibody P10 bound 500 % as well with IL2-lectin deficient-RTB as with plant RTB. Antibody to IL2 bound the hybrid molecule 20% as well as with recombinant human IL2 on a molar basis.

Immunoblots demonstrated reactivity with the same 52 kDa band using anti-RTB or anti-IL2 antibodies. No weaker bands at lower molecular weight were observed with either set of antibodies, suggesting the partial proteolysis found with IL2-"wild-type" RTB was not present with the lectin-deficient chimeras.

Reassociation with RTA. Two preparations of fusion toxin heterodimer were made. Under the reaction conditions (10^{-6} M of IL2-lectin-deficient RTB and 3 x 10^{-6}M of plant RTA, 0.1 M triethylamine/0.1 M sodium phosphate pH7, room temperature, room air), 83% reassociation was observed in one reaction and 78 % reassociation was seen in the other. The results from the sandwich ricin ELISA were confirmed by immunoblots with antibodies to RTB, RTA and IL2.

Lectin activity and IL2R binding of the heterodimer. ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]-RTA bound immobilized asialofetuin 0.3% as well as plant ricin. The ricin Kd was 4 x 10^{-9} M and the IL2 fusion toxin Kd was 1.2 x 10^{-7} M. Specificity for high affinity IL2 receptor was demonstrated on a live cell immunofluorescence assay. The IL2 fusion toxin bound to HUT102,

YT2C2, MT-1 but did not bind CEM or KB cells. Binding to HUT102 cells was inhibited by IL2 but not asialofetuin.

Cell cytotoxicity to mammalian cells. Cytotoxicities of fusion heterodimer and plant ricin for different cell lines is shown in Table 2. Ricin was uniformly toxic to all five cell lines tested with IC_{50} values of 2 - 3.5 x 10^{-12} M. IL2-wild-type RTB-RTA was also toxic to all cell lines in the absence of lactose with IC_{50} values of 2 -4 x 10^{-12} M. IL2-RTB[W37S/Y248H]-RTA showed moderate specificity with IC_{50} values of 4 x 10^{-12} M on HUT102 cells, 1.8 x 10^{-10} M on CEM cells and 2 x 10^{-10} M on KB cells. In contrast, the IL2-triple-site RTB mutant-RTA had improved specificity with IC_{50} values of 5 x 10^{-12} M on HUT102 cells, 1 x 10^{-9} M on CEM cells and 6 x 10^{-10} M on KB cells. The *in vitro* therapeutic window (the ratio of the IC_{50} of receptor negative cells to the IC_{50} of receptor positive cells) was 1 for IL2-wild-type RTB-RTA, 50 for IL2-RTB[W37S/Y248H]-RTA and 120 - 200 for IL2-RTB[W37S/Y248H/Y78H]-RTA.

IL2 receptor-mediated cell toxicity was tested by blocking experiments with excess IL2 or lactose. Excess IL2 reduced IL2-triple-site RTB mutant-RTA toxicity towards HUT102 cells by 1,000-fold (IC_{50} was 1 x 10^{-8} M with IL2 and 1 x 10^{-11} M without IL2). In contrast, excess lactose had minimal effect on IL2-lectin-deficient ricin cytotoxicity (IC_{50} was 1.6 x 10^{-11} M with lactose and 1.4 x 10^{-11} M without lactose).

Synthesis of an IL2R targeted ricin fusion protein for preclinical and clinical development requires adequate yields, simple purification, adequate stability at room temperature and 37°C, and selective toxicity to IL2R bearing lymphocytes.

The IL2 triple-site mutant RTB fusion protein was obtained at 50% purity in good yields of 0.75 mg/liter culture. This compares with 1 mg/liter for IL2 wild-type RTB and 0.34 mg/liter IL2 double-site mutant RTB fusion protein. The IL2 triple-site mutant RTB molecules reacted with antibodies to IL2 and RTB both by ELISA and Western blots. These results are evidence of proper folding of both the IL2 and RTB domains. Further, the protein was secreted into the insect cell medium, and purification was accomplished by a one-step immunoaffinity absorption. This contrasts with the requirement for denaturation and refolding for bacterial toxin fusion proteins and chemical derivatization and conjugation for immunotoxins.

The toxophore domain of ricin (RTA) was added by simply mixing with the IL2 triple-site mutant RTB at 10^{-6} M. Extensive ionic and hydrophobic bonds in the RTA-RTB interface promote reassociation and disulfide bond formation (13). The observation of 80% reassociation compares favorably with the 60% reassociation for IL2 wild-type RTB-RTA (54), 55% reassociation for IL2 double-site mutant RTB-RTA and 50% reassociation for plant RTB-RTA under identical conditions (53). The heterodimers were stable at high dilution (10-12 M) suggesting formation of the disulfide bond between RTA Cys-259 and RTB Cys-4 (55).

Binding specificity of the lectin-deficient heterodimer was demonstrated in both ELISA and cell immunofluorescence formats. The fusion toxin displayed 0.3% binding to immobilized asialofetuin. The Kd was 1.2×10^{-7} M versus 4×10^{-9} M for plant ricin. This weak binding compares to 1% binding (Kd = 4×10^{-7} M) for IL2-RTB[W37S/Y248H]-RTA and 59% binding (Kd = 7×10^{-9} M) for IL2-wild type RTB-RTA. The low level binding observed in the ELISA is near the limits of detection in this assay (lower limit 0.1% relative to wild type ricin or Kd = 4×10^{-6} M). Nevertheless, the small residual binding of

the 1α, 1β, 2γ triple mutant fused to IL2 appears to be real and due to incomplete inactivation of one or more sites. Subdomain 1α mutation W37S reduced sugar binding avidity 4-fold, while other 1α subdomain mutations (K40M and K40M/N46G) yielded proteins with 7-8-fold reductions in asialofetuin avidity (40). The W37S mutation was used in the IL2-triple-site mutant because of its much better yields.

IL2 triple-site RTB mutant-RTA bound to cells possessing low, intermediate and high affinity IL2R. The lack of detectable binding to other human cell lines which still have cell surface galactosides may be due to the insensitivity of the immunofluorescence assay. a two and one-half log reduction in sugar binding may be beneath the immunofluorescence detection limit. Binding to cell lines was blocked by IL2 but not asialofetuin.

The most important property of an IL2-lectin-deficient ricin fusion protein is its selective cytotoxicity to IL2R expressing cells. Three previous studies suggested that targeted ricin molecules in which the galactose-binding sites were removed genetically or chemically lose critical intracellular intoxication functions and cannot kill cells (33-35). Goldmacher used antibody conjugates to ricin with one, two or three affinity cross-linkers (33). The triply cross-linked molecules lacked sugar-binding and were unable to intoxicate antigen-bearing cells. Newton reassociated *Xenopus laevis* oocyte-derived RTB with modifications of two lectin sites (K40M/N46G/N255G) with plant RTA (34). The lectin-deficient heterodimer was nontoxic to mouse macrophages in the presence of lactose, even though binding and internalization was mediated by binding to mannose receptors. Finally, Youle attached mannose-6-phosphate to tyrosyl acetylated ricin and measured cytotoxicity to mannose-6-phosphate receptor expressing fibroblasts in the presence of lactose (35). The lectin-deficient ricin conjugate again had reduced

cell cytotoxicity, although cell binding and entry was mediated by non-galactoside mechanisms.

The IL2-lectin-deficient fusion toxin was selectively cytotoxic to hematopoietic neoplastic cell lines with the heterotrimeric high affinity IL2R. The molecules were less toxic to cells with intermediate or low affinity IL2R, and nontoxic to cells without IL2R. These results are similar to those seen with IL2-PE40[35] but distinct from those seen with $DAB_{389}IL2$. The latter molecule fails to intoxicate low affinity IL2Rα,γ cells perhaps due to steric effects of the N-terminal toxin moiety. As a note of caution, fresh leukemic blasts often display lower levels of IL2Rα and IL2Rβ than cell lines and may show lowered sensitivity to the ricin fusion molecule (23).

Competition experiments with excess ligand demonstrated that IL2, but not lactose, inhibited cell cytotoxicity of the IL2-triple-site RTB mutant-RTA. Thus, the fusion toxin needs IL2R binding for cell intoxication.

EXAMPLE IV: Studies of mannose receptor mediated cell cytotoxicity of ricin fusion protein immunotoxins containing a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains.

Preparation of toxins. Recombinant baculovirus encoding the gp67A leader and the RTB mutant (W37S/Y78H/Y248H) was prepared as previously described and used to infect Sf9 insect cells at an moi of 5-10 in EXCELL400 medium (JRH Scientific, Lexena, KS) supplemented with 25 mM lactose. W37SiY78H/Y248H RTB protein was purified from day 6 post-infection cell supernatants by, sequentially, vacuum dialysis, dialysis into 50 mM NACL, 25 mM Tris HCL pH 8, 1 mM EDTA, 0. I% sodium azide, and 25 mM lactose, binding and elution from a P2 monoclonal antibody anti-RTB azlactone

functionality bis-acrylamide affinity matrix using 0. I M triethylamine HCL (pH 11) elution buffer and neutralization with 11 M NAPO, pH 4. Protein was quantitated by P2 antibody ELISA and reassociated overnight at room temperature with a three-fold molar excess of plant RTA (Inland Laboratories, Austin, TX). Heterodimer concentration was determined by a sandwich ELISA employing P2 monoclonal anti-RTB antibody capture and biotinylated αBR12 anti-RTA monoclonal antibody detection. Plant ricin was obtained from Inland Laboratories.

Glycosylation of W37S/Y78H/Y248H RTB. Using a previously reported procedure (53), two T-25 flasks were inoculated with 5 x 10^6 Sf9 cells, 10 ml of TMNFH media and recombinant baculovirus encoding RTB[W37S/Y78H/Y248H] at an moi of 1. After 48 hours, medium in one flask was changed to TMNFH plus 10 μg/ml tunicamycin. After 60 hours, the medium of both flasks was changed to Grace's medium without methionine supplemented with 10% dialyzed fetal bovine serum. Again, 10 μg/ml tunicamycin was added to one flask. After two hours, 250 μCi ^{35}S-methionine (1000 mci.mmol) was added to each flask. Four hours later, the medium was removed and replaced with PBS. One of the flasks again contained 10 μg/ml tunicamycin. After an additional six hours of incubation, the cells and supernatants from each flask were harvested. The cells were suspended in 150 mM NaCl/1% NP40/0.5% DOC/0.1% SDS/50 mM Tris pH 8/25 mM lactose (RIPAL). After freezing and thawing, cell extracts were centrifuged at 22,000g for 10 minutes. Pansorbin fixed *Staphyloccocus aureus* Cowan strain was prewashed with 3% BSA in PBS and then incubated with cell extracts to remove non-specifically-binding labeled materials. The metabolically-labeled solutions were then reacted with Pansorbin pretreated with rabbit anti-ricin antibody. The Pansorbin was then pelleted and washed with RIPAL twice and RIPAL plus 500 mM NaCl once. The pellets were then boiled in reducing

sample buffer and the samples run along with prestained low molecular standards on a 15% SDS polyacrylamide gel. The gel was fixed briefly with 10% acetic acid, soaked for one hour in Enlightening, dried, exposed to x-ray film with enhancing screens for 1-3 days at -70°C and developed.

Macrophage cells. Mouse peritoneal macrophages were prepared using 12 week old female Balb/c mice house in an IACUC approved facility. Briefly, mice were injected intraperitoneally with 1.5 ml Brewer's thioglycollate medium (Becton Dickinson Microbiology Systems). After five days, mice were sacrificed. The mice were then injected into their thigh fatty deposits with 10 ml of cold HEPES buffered Hank's balanced salt solution with 10 U/ml heparin with 11% BSA. After injection, the mice were gently shaken and the injected medium withdrawn. The cell suspension was centrifuged at 1,000x g for 10 minutes and the cell pellet resuspended in 10 ml of RPMI1640 with 10% fetal calf serum and 0.01 M HEPES. Cells were then diluted to 2×10^6/ml and seeded to 96 well plates at 100 µl/well. After incubation for two hours at 37°C/5% CO_2, the plates were washed three times with warm Hank's balanced salt solution buffered with HEPES. After washing, 100 µl/well leucine-free RPMI1640 medium with 10% dialyzed fetal calf serum was added. 35 mm petri dishes were treated similarly except 2 ml of cells representing 2×10^5 cells/dish was used.

The J774E mouse macrophage cell line (56) was cultured in α-MEM with 10% fetal calf serum, 60 µ thioguanine and transferred from flasks to wells and dishes by exposure to trypsin EDTA (Gibco). Cells were plated at 2×10^4 cells/well in 96 well plates and 2×10^5 cells/35 mm dish, and incubated a further 24 hours at 37°C/5% $C0_2$ prior to assay.

MMR61 rat fibroblasts transfected with mouse mannose receptor cDNA were grown in Dulbecco's MEM containing 10% fetal calf serum and 400 ug/ml G418 (57). Cells were split and transferred to wells and dishes again by trypsinization and incubated a further 24 hours prior to assay.

KB human epidermoid carcinoma cells obtained from the American Type Culture Collection (Rockville, MD) were grown in Dulbecco's MEM with 10% fetal calf serum (58). Cells were removed from flasks by trypsin treatment and seeded for experiments identically to the other cell types.

Cytotoxicity assays. After two washes with Hank's balanced salt solution, the mouse peritoneal macrophage cells in each well of the 96 well plate were resuspended in 100 µl of leucine-free RPMI1640 with 10% dialyzed fetal calf serum and different concentrations of toxin. Twelve different concentrations of toxin were used in each experiment. Sets of wells in each experiment contained 60 mM lactose and/or 1 mg/ml yeast mannan. After 20 hours, 50 µl of leucine-free media containing 1 uCi ^{3}H-leucine (Amersham, 300 mCi/mmol) with or without added lactose and/or mannan was added to the wells. The cells were again incubated four hours at 37°C/5% CO_2 and then harvested with a Skatron cell harvester onto glass fiber filter mats. Filters were dried and counted in Econofluor liquid scintillation fluid in a LKB liquid scintillation counter. The IC_{50} was determined for each toxin/cell type/medium condition as the toxin concentration that reduced protein synthesis to 50% of control. Each assay was performed in quadruplicate. The mannose receptor directed toxicity was quantitated by the ratio of the toxin IC_{50} in the presence of lactose plus mannan to the IC_{50} in the presence of lactose alone. KB cells were assayed identically. J774E and MMR61 were assayed identically except leucine-free DMEM medium was used instead of RPMI1640.

Immunofluorescence assay. Mouse peritoneal macrophages, J774E, MMR61 and KB cells were attached for 24 hours at 37°C in 5%$C0_2$ to petri dishes, fixed in 3.7% formaldehyde in PBS (15 minutes), washed with 2 mg/ml BSA in PBS and 0.1% saponin and incubated in PBS plus BSA with rabbit anti-mouse mannose receptor antibody (56) for 30 minutes at 4°C. The cells were rewashed with PBS and reacted with goat anti-(rabbit Ig) conjugated to rhodamine (Jackson ImmunoResearch, West Grove, PA) at 25 ug/ml with 0.1% saponin for 30 minutes at 23°C. After a final wash with PBS, the cells were fixed in 3.7% formaldehyde in PBS, mounted under a #1 coverslip in glycerol-PBS (90:10) and examined using a Zeiss Axioplan epifluorescence microscope (63 x, N.A. 1.4 planapochromat objective). Fluorescence images were recorded using Tri-X film and negatives were digitized and edited using Adobe Photoshop software and a PowerMac 8500/120 computer. Relative intensities for the brightness of mannose receptor reactions in the different cell types were, respectively, for K-B, for J774E cells, and (+) for MMR61 and mouse peritoneal macrophages [scale (-) - (++++)].

Triple-site mutant RTB glycosylation. W37S/Y78H/Y248H RTB was immunoprecipitated from Sf9 infected cell extracts after metabolic labeling with ^{35}S-methionine in the presence and absence of tunicamycin. Autoradiographs of reducing SDS polyacrylamide gels showed bands at 33 kilodaltons molecular weight in the absence of tunicamycin and 29 kilodaltons molecular weight in the presence of tunicamycin corresponding to 4 kilodaltons of attached sugars per molecule.

Triple-site mutant RTB-RTA heterodimer. Two preparations of W37S/Y78H/Y248H RTB-RTA were made each at 40 ug/ml concentration. Each showed 0. 1% asialofetuin binding avidity relative to plant ricin.

Mannose receptor-targeted ricin toxicity. Ricin-related proteins in the presence of excess lactose bind and intoxicate mannose receptor-deficient cells only weakly. The IC_{50} of ricin on KB cells in the presence of lactose was 3 x 10^{-9} M. The IC_{50} of the triple-site mutant RTB-RTA with lactose was 3 x 10^{-8} M.

In contrast, cells with mannose receptor are sensitive to ricin proteins even in the presence of excess lactose due to mannose-terminated oligosaccharides on plant and insect-derived ricin. The IC_{50}'s of ricin with lactose on mouse peritoneal macrophages, J774E cells and MMR61 rat fibroblasts transfected with mouse mannose receptor were 8 x 10^{-11} M, 1 x 10^{-10} M, and 8 x 10^{-10} M, respectively. Similarly, the triple-site mutant RTB-RTA with lactose showed IC_{50}'s on mouse peritoneal macrophages, J774E cells and MM61 cells of 1 x 10^{-9} M, 3 x 10^{-10} M, and 7 x 10^{-9} M, respectively. Thus, the receptor-positive cells were 4 - 36 fold more sensitive to ricin and 30-100 fold more sensitive to triple-site mutant RTB-RTA heterodimer than receptor-negative cells.

Lower efficacy of cell intoxication by mannose receptor pathway. Ricin binds cell surface galactosides in the absence of lactose and intoxicates cells with an IC_{50} of 1 **X** 10^{-12}, 11 x 10^{-11} M and 2 x 10^{-11} M on mouse peritoneal macrophages, J774E cells and MMR61 cells, respectively. Thus, ricin was 10-80 fold more potent in the absence of lactose. The lectin-deficient mutant ricin had minimal residual galactoside mediated cytotoxicity with IC_{50}'S of 5 x 10^{-10} M, 7 x 10^{-10} M, and 3 x 10^{-9} M on mouse peritoneal macrophages, J774 E cells and MMR61 cells, respectively. This was a 0.3 - 2 fold relative potency to mutant heterodimer in the presence of lactose.

Competition of mannose receptor-mediated toxin uptake by mannan. The D-mannose receptor negative KB cells showed no inhibition of ricin cytotoxicity by mannan versus control (IC_{50} Of 9 x 10^{-12M} for both) or mannan plus lactose versus lactose alone (IC_{50} of 3 x 10^{-8}M for both). Similarly, KB cells showed no effects of mannan on lectin deficient ricin toxicity in the absence of lactose (IC_{50} of 1 x 10^{-8} M for both) or presence of lactose (IC_{50} of 3 x 10^{-8} M for both).

In contrast, ricin toxicity to all three mannose receptor positive cell lines in the presence of lactose was inhibited by mannan. Mannan increased the IC_{50} to 2 x 10^{-10} M, 3 x 10^{-9} M, and 3 x $^{10-9}$M for mouse peritoneal macrophages, J774 E cells, and MMR61 cells, respectively. This represented a 3 - 30 fold reduction in toxicity. Lectin-deficient ricin behaved like ricin with significant mannan inhibition of toxicity on these cell lines. Mannan increased the IC_{50} to 5 x 10^{-9} M, 2 x 10^{-8} M, and 3 x 10^{-8} M for mouse peritoneal macrophages, J774E cells and MMR61 cells, respectively. This yielded a 4 - 50 fold reduction in toxicity.

Immunologic detection of D-mannose receptor. Rabbit antibody to mouse mannose receptor reacted strongly with both surface and intracellular sites in J774E and MMR61 cells lines and many of the adherent cells from thioglycollate treated mouse peritoneal fluid cells. No binding was observed with the KB human epidermoid carcinoma cells. Localization was seen not only in an intracellular granular organelle pattern, but also in association with the ruffled border of cells, consistent with a cell surface distribution.

The mechanism by which internalized ricin toxin is transported to a translocation competent intracellular organelle is unknown. Plant ricin and insect-derived RTBRTA heterodimers have mannose-rich N-glycans and thus

may be bound and internalized by cells possessing the D-mannose receptor in the presence of excess lactose to block binding to cell surface galactosides. These studies demonstrate that RTB intracellular galactose binding activity is not required for ricin toxicity. Surprisingly, the lectin-deficient ricin retained cytotoxic potency implying either that RTB lectin activity is not required for intracellular transport and cell intoxication or that the small amount of residual lectin function (0.1% of normal) is sufficient for critical intracellular routing.

The lower potency of mannose receptor-mediated toxicity for both lactose blocked ricin and lectin-deficient ricin may be due to reduced surface receptor content or lower avidity of cell surface binding for the D-mannose receptors and may not reflect altered intracellular processing. The number of galactosyl-terminated cell surface glycoprotein receptors for ricin has been reported to be about 10^7/mammalian cell (59) versus 10^5/cell for D-mannose receptors (56). Avidities for each were similar with Ka's of 10^9 M^{-1} (56,59).

The residual cell toxicity of ricin-related molecules in the presence of mannose and lactose was 10^{-9} - 10^{-8} M. This toxicity is ten-fold higher than RTA alone and probably reflects incomplete competition at 37°C by the soluble lactose and yeast mannan.

The greater sensitivity of J774E cells relative to MMR61 or mouse peritoneal macrophages may be due to higher cell surface mannose receptor density on J774E cells or different intracellular metabolism. The immunofluorescence assay suggests higher receptor content is the cause for the J774E sensitivity difference.

The observation of efficient cell killing in the absence of RTB lectin function for mannose receptor has also been documented for the IL2 receptor

using an insect-derived lectin-deficient IL2 ricin fusion molecule. RTB enhancement for other receptors may be due to inefficient internalization by the ligand-receptor complex or misrouting intracellularly away from a translocation competent compartment (61,62).

Exploration of the intracellular steps in ricin intoxication remains an important avenue for defining molecular signals for routing of soluble intracellular polypeptides. This study provides a model system for examining other ligand-receptors and combined with the new molecular tags such as green fluorescent protein (63), may permit better understanding of important chemical reactions that mediate vectorial transport inside cells.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Although the present invention has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

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Table 1. Properties of insect-derived ricin proteins*

Protein	Galactose binding (kD asialofetuin)	Cell cytotoxicity (IC_{50} HUT102 cells)
Ricin	4×10^{-9} M	4×10^{-12} M
RTB[Y248H]-RTA	2×10^{-8} M	8×10^{-11} M
RTB[W37S/Y248H]-RTA	2×10^{-7}M	2×10^{-10} M
RTB[W37S/Y78H/Y248H]-RTA	6×10^{-6}M	5×10^{-9} M
RTA	$> 10^{-5}$ M	1×10^{-7}M

*Galactose binding quantitated based on RTB binding to immobilized asialofetuin. Cell cytotoxicity measured by 20 hour exposure to toxin in leucine-free media followed by 4 hour pulse with ^{3}H-leucine and filtration on glass fibre mats. Ricin and RTA obtained from castor beans.

Table 2. Cell cytotoxicity of ricin fusion proteins

Cell line	IC_{50}(M)			
	RICIN	IL2-W.T. RICIN	IL2-D.S. RICIN	IL2-T.S. RICIN
HUT102	2×10^{-12}	2×10^{-12}	4×10^{-12}	5×10^{-12}
CEM	3.5×10^{-12}	4×10^{-12}	1.8×10^{-10}	1×10^{-9}
KB	2×10^{-12}	2×10^{-12}	2×10^{-10}	6×10^{-10}
MT-1	2×10^{-12}	3×10^{-12}	1×10^{-11}	8×10^{-11}
YT2C2	2×10^{-12}	4×10^{-12}	9×10^{-12}	1×10^{-11}

*Cell cytotoxicity assays performed in triplicate as described in text. IC_{50} is the concentration of toxin reducing protein synthesis by 50% after 24 hour incubation. IL2-W.T. RICIN= IL2-wild-type RTB-RTA; IL2-D.S. RICIN=IL2-RTB[W37S/Y248H]-RTA; IL2-T.S. RICIN=IL2-RTB[W37S/Y248H/Y78H]-RTA.

What is claimed is:

1. A plant holotoxin fusion protein comprising a plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains and a ligand specific for a cell surface receptor.

2. The fusion protein of claim 1, wherein the modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains consists of an amino acid substitution.

3. The fusion protein of claim 2, wherein the amino acid substitution is a substitution of an amino acid having an aromatic ring with an amino acid lacking an aromatic ring.

4. A ricin toxin B chain fusion protein comprising a ricin toxin B chain having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains and a ligand specific for a cell surface receptor.

5. The fusion protein of claim 4, wherein the modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains consists of an amino acid substitution.

6. The fusion protein of claim 5, wherein the amino acid substitution is a substitution of an amino acid having an aromatic ring with an amino acid lacking an aromatic ring.

7. The fusion protein of claim 6, wherein the amino acid substitutions consist of a W to S substitution at amino acid position 37 in the 1α subdomain, a Y to H substitution at amino acid position 248 in the 1β subdomain and a Y to H substitution at position 78 in the 2γ subdomain.

8. The fusion protein of claim 4, wherein the ligand is interleukin-2.

9. The fusion protein of claim 4, wherein the ligand is granulocyte/macrophage colony stimulating factor.

10. The fusion protein of claim 4, wherein the ligand is an antibody to CD3.

11. The fusion protein of claim 4, wherein the ligand is an antibody to GD2.

12. The fusion protein of claim 4, wherein the ligand is epidermal growth factor.

13. A ricin toxin B chain fusion protein comprising a moiety consisting of a ricin toxin B chain comprising a W to S substitution at amino acid position 37 in the 1α subdomain, a Y to H substitution at amino acid position 248 in the 1β subdomain and a Y to H substitution at position 78 in the 2γ subdomain and a moiety consisting of a ligand specific for a cell surface receptor.

14. The fusion protein of claim 13, wherein the ligand is interleukin-2.

15. The fusion protein of claim 13, wherein the ligand is granulocyte/macrophage colony stimulating factor.

16. The fusion protein of claim 13, wherein the ligand is an antibody to CD3.

17. The fusion protein of claim 13, wherein the ligand is an antibody to GD2.

18. The fusion protein of claim 13, wherein the ligand is epidermal growth factor.

19. A ricin fusion protein immunotoxin comprising the ricin toxin B chain fusion protein of claim 13 associated with a ricin toxin A chain.

20. The ricin fusion protein immunotoxin of claim 19 in a pharmaceutically acceptable carrier.

21. A nucleic acid encoding the fusion protein of claim 1.

22. A vector comprising the nucleic acid of claim 21.

23. A host comprising the vector of claim 22.

24. A nucleic acid encoding the fusion protein of claim 4.

25. A vector comprising the nucleic acid of claim 24.

26. A host comprising the vector of claim 25.

27. A plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains.

28. The plant holotoxin of claim 27, having at least a one thousand fold reduction in sugar binding and at least a one hundred fold reduction in toxicity in mice.

29. The plant holotoxin of claim 27, wherein the modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains is an amino acid substitution.

30. The plant holotoxin of claim 29, wherein the amino acid substitution consists of substitution of an amino acid having an aromatic ring residue with an amino acid lacking an aromatic ring residue.

31. A ricin toxin B chain having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains.

32. The ricin toxin B chain of claim 31, having at least a one thousand fold reduction in sugar binding, associating normally with ricin A toxin and having at least a one hundred fold reduction in toxicity in mice.

33. The ricin toxin B chain of claim 31, wherein the modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains is an amino acid substitution.

34. The ricin toxin B chain of claim 33, wherein the amino acid substitution consists of substitution of an amino acid having an aromatic ring residue with an amino acid lacking an aromatic ring residue.

35. The ricin toxin B chain of claim 34, wherein the amino acid substitutions consist of a W to S substitution at amino acid position 37 in the 1α subdomain, a Y to H substitution at amino acid position 248 in the 1β subdomain and a Y to H substitution at position 78 in the 2γ subdomain.

36. A ricin fusion protein immunotoxin comprising the ricin toxin B chain fusion protein of claim 4 associated with a ricin toxin A chain.

37. The ricin fusion protein immunotoxin of claim 36 in a pharmaceutically acceptable carrier.

38. A nucleic acid encoding the plant holotoxin of claim 27.

39. A vector comprising the nucleic acid of claim 38.

40. A host comprising the vector of claim 39.

41. A nucleic acid encoding the ricin toxin B chain of claim 31.

42. A vector comprising the nucleic acid of claim 41.

43. A host comprising the vector of claim 42.

44. A method of constructing a ricin fusion protein immunotoxin comprising:

a) expressing the nucleic acid in the vector of claim 25 in a eukaryotic cell expression system to produce a fusion protein;

b) isolating and purifying the fusion protein of step a); and

c) contacting the fusion protein of step b) with a ricin toxin A chain under conditions which permit the association of the fusion protein with the ricin toxin A chain.

45. A method of treating a cancer or an autoimmune disease in a patient diagnosed with a cancer or an autoimmune disease comprising:

a) constructing the ricin fusion protein immunotoxin of claim 44, wherein the ligand is specific for a particular cell surface receptor present only on the surfaces of the cancer cells or on the surfaces of the cells causing the patient's autoimmune disease; and

b) administering the ricin fusion protein immunotoxin in a pharmaceutically acceptable carrier to the patient, whereby the ricin fusion protein immunotoxin treats the patient's cancer or autoimmune disease.

46. The method of claim 45, wherein the cancer is a human leukemia or lymphoma having cancer cells expressing interleukin-2 receptors on the surfaces and the ligand is interleukin-2.

47. The method of claim 45, wherein the autoimmune disease is graft-versus-host disease and the ligand is an antibody to CD3.

48. The method of claim 45, wherein the cancer is acute myelogenous leukemia and the ligand is granulocyte/macrophage-colony stimulating factor.

49. The method of claim 45, wherein the cancer is melanoma/neuroblastoma and the ligand is an antibody to GD2.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/19577

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/415 C12N15/62 A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 89 04839 A (GENETICS INST) 1 June 1989 see the whole document ---	1-49
X	FRANKEL A. ET AL.,: "Double-site ricin B chain mutants retain galactose binding" PROTEIN ENGINEERING, vol. 9, no. 4, - April 1996 pages 371-379, XP002059508 see whole document, esp. discussion ---	1-7,13, 19-45
Y	FRANKEL A. ET AL.,: "IL-2 ricin fusion toxin is selectively cytotoxic in vitro to IL2 receptor-bearing tumor cells" BIOCONJ. CHEM., vol. 6, no. 6, - 1995 pages 666-672, XP002059509 cited in the application see whole document, esp. discussion --- -/--	1-8,13, 14,19-46

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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1

Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/19577

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 89 01037 A (CETUS CORP) 9 February 1989 see claims and page 11, line 32 ff.	1-8,13, 14,19-46
A	SPHYRIS N. ET AL.,: "Mutational analysis of the ricin lectin B-chains" J. BIOL. CHEM., vol. 270, no. 35, - 1 September 1995 pages 20292-20297, XP002059510 see the whole document	1-49
A	RUTENBER E. & ROBERTUS J. D. : "Structure of Ricin B-chain at 2.5A resolution" PROTEINS, vol. 10, - 1991 pages 260-269, XP002059515 see the whole document	1-49
A	WILLIAMS D. P. ET AL.,: "Diptheria toxin receptor binding domain substitution with interleukin-2: genetic construction and properties of a diphteria toxin-related interleukin-2 fusion protein" PROTEIN ENGINEERING, vol. 1, no. 6, - 1987 pages 493-498, XP002059511 see the whole document	1-49
P,X	FRANKEL A. E. ET AL.,: "IL2 fused to lectin-deficient ricin is toxic to human leukemia cells expressing the IL2 receptor" LEUKEMIA, vol. 11, - January 1997 pages 22-30, XP002059512 see the whole document	1-8,13, 14,19-46
P,X	BURBAGE C. ET AL.,: "Rcin fusion toxin targeted to the human granulocyte-macrophage colony stimulating factor receptor is selectively toxic to acute myeloid leukemia cells" LEUKEMIA RESEARCH, vol. 21, no. 7, - July 1997 pages 681-690, XP002059513 see the whole document	1-7,9, 13,15, 19-45,48
P,X	FRANKEL A. E. ET AL.,: "Ricin toxin contains at least three galactose-binding sites located in B chain subdomains 1alpha,1beta, and 2gamma" BIOCHEMISTRY, vol. 35, - 26 November 1996 pages 14749-14756, XP002059514 see the whole document	1-8,13, 14,19-46

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/19577

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 45-49
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 45-49 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/19577

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 8904839 A	01-06-89	EP 0341304 A	15-11-89
		JP 2502287 T	26-07-90
WO 8901037 A	09-02-89	AU 2136788 A	01-03-89